jc586 U.S. PTO

02/08/00

2-10-00

PTO
U.S.
jc526 09/500135
02/08/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Date: __February 8, 2000__

File No. __A-68893/DJB/DAV__

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Transmitted herewith for filing is the patent application of Inventor(s):

David Estell and Fiona Harding

For: **MUTANT PROTEINS FOR ALTERING THE IMMUNOGENIC RESPONSE IN HUMANS AND METHODS OF MAKING AND USING THE SAME**

Enclosed are also:

___ Sequence Listing w/diskette

_XX_ __27__ sheets of drawing, Formal ___ , Informal _X_

___ An Assignment of the invention to: _____

___ Cost of recording to be charged to Deposit Account No. 06-1300 (Order No. A- /)

___ Power of Attorney by Assignee & Exclusion of Inventor Under 37 CFR 1.32

___ Declaration for Patent Application

___ Small Entity Status Declaration Under 37 CFR _____

	(Col. 1) NO. FILED	(Col. 2) NO. EXTRA	SMALL ENTITY RATE	FEE	OTHER THAN SMALL ENTITY RATE	FEE
BASIC FEE				$345		$690
TOTAL CLAIMS	- 20 = 0	0	x 09 =	$	x 18 =	$
INDEP CLAIMS	- 3 = 0	0	x 39 =	$	x 78 =	$
MULTIPLE DEPENDENT CLAIM PRESENTED			+130 =	$	+260 =	$
If the difference in Col 1 is less than zero, enter "0" in Col 2			TOTAL	$	TOTAL	$

_X_ The filing fee is **not enclosed.**

_X_ This application is a continuation-in-part of Serial No. __09/060,872__ filed __April 15, 1998__.

Respectfully submitted,

Dolly A. Vance, Reg. No. 39,054

FLEHR HOHBACH TEST ALBRITTON & HERBERT LLP
Four Embarcadero Center, Suite 3400
San Francisco, California 94111-4187
Telephone: (415) 781-1989 Fax: (415) 398-3249

PROTEINS PRODUCING AN ALTERED IMMUNOGENIC RESPONSE AND METHODS OF MAKING AND USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation in part of USSN 09/060,872, filed on April 15, 1998, which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Proteins used in industrial, pharmaceutical and commercial applications are of increasing prevalence. As a result, the increased exposure due to this prevalence has been responsible for some safety hazards caused by the sensitization of certain persons to those peptides, whereupon subsequent exposure causes extreme allergic reactions which can be injurious and even fatal. For example, proteases are known to cause dangerous hypersensitivity in some individuals. As a result, despite the usefulness of proteases in industry, e.g., in laundry detergents, cosmetics, textile treatment etc., and the extensive research performed in the field to provide improved proteases which have, for example, more effective stain removal under detergency conditions; the use of proteases in industry has been problematic due to their ability to produce a hypersensitive allergenic response in some humans.

Much work has been done to alleviate these problems. Among the strategies explored to reduce immunogenic potential of protease use have been improved

production processes which reduce potential contact by controlling and minimizing workplace concentrations of dust particles or aerosol carrying airborne protease, improved granulation processes which reduce the amount of dust or aerosol actually produced from the protease product, and improved recovery processes to reduce the level of potentially allergenic contaminants in the final product. However, efforts to reduce the allergenicity of protease, per se, have been relatively unsuccessful. Alternatively, efforts have been made to mask epitopes in protease which are recognized by immunoglobulin E (IgE) in hypersensitive individuals (PCT Publication No. WO 92/10755) or to enlarge or change the nature of the antigenic determinants by attaching polymers or peptides/proteins to the problematic protease.

When an adaptive immune response occurs in an exaggerated or inappropriate form, the individual experiencing the reaction is said to be hypersensitive. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately and sometimes cause inflammatory reactions and tissue damage. They can be provoked by many antigens; and the cause of a hypersensitivity reaction will vary from one individual to the next. Hypersensitivity does not normally manifest itself upon first contact with the antigen, but usually appears upon subsequent contact. One form of hypersensitivity occurs when an IgE response is directed against innocuous environmental antigens, such as pollen, dust-mites or animal dander. The resulting release of pharmacological mediators by IgE-sensitized mast cells produces an acute inflammatory reaction with symptoms such as asthma or rhinitis.

Nonetheless, a strategy comprising modifying the IgE sites will not generally be successful in preventing the cause of the initial sensitization reaction. Accordingly, such strategies, while perhaps neutralizing or reducing the severity of the subsequent hypersensitivity reaction, will not reduce the number or persons actually sensitized. For example, when a person is known to be hypersensitive to a certain antigen, the general, and only safe, manner of dealing with such a situation is to

isolate the hypersensitive person from the antigen as completely as possible. Indeed, any other course of action would be dangerous to the health of the hypersensitive individual. Thus, while reducing the danger of a specific protein for a hypersensitive individual is important, for industrial purposes it would be far more valuable to render a protein incapable of initiating the hypersensitivity reaction in the first place.

T-lymphocytes (T-cells) are key players in the induction and regulation of immune responses and in the execution of immunological effector functions. Specific immunity against infectious agents and tumors is known to be dependent on these cells and they are believed to contribute to the healing of injuries. On the other hand, failure to control these responses can lead to auto aggression. In general, antigen is presented to T-cells in the form of antigen presenting cells which, through a variety of cell surface mechanisms, capture and display antigen or partial antigen in a manner suitable for antigen recognition by the T-cell. Upon recognition of a specific epitope by the receptors on the surface of the T-cells (T-cell receptors), the T-cells begin a series of complex interactions, including proliferation, which result in the production of antibody by B-cells. While T-cells and B-cells are both activated by antigenic epitopes which exist on a given protein or peptide, the actual epitopes recognized by these mononuclear cells are generally not identical. In fact, the epitope which activates a T-cell to initiate the creation of immunologic diversity is quite often not the same epitope which is later recognized by B-cells in the course of the immunologic response. Thus, with respect to hypersensitivity, while the specific antigenic interaction between the T-cell and the antigen is a critical element in the initiation of the immune response to antigenic exposure, the specifics of that interaction, i.e., the epitope recognized, is often not relevant to subsequent development of a full blown allergic reaction.

PCT Publication No. WO 96/40791 discloses a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity using polyalkylene oxide as a starting material.

PCT Publication No. WO 97/30148 discloses a polypeptide conjugate with reduced allergenicity which comprises one polymeric carrier molecule having two or more polypeptide molecules coupled covalently thereto.

PCT Publication No. WO 96/17929 discloses a process for producing polypeptides with reduced allergenicity comprising the step of conjugating from 1 to 30 polymolecules to a parent polypeptide.

PCT Publication No. WO 92/10755 discloses a method of producing protein variants evoking a reduced immunogenic response in animals. In this application, the proteins of interest, a series of proteases and variants thereof, were used to immunize rats. The sera from the rats was then used to measure the reactivity of the polyclonal antibodies already produced and present in the immunized sera to the protein of interest and variants thereof. From these results, it was possible to determine whether the antibodies in the preparation were comparatively more or less reactive with the protein and its variants, thus permitting an analysis of which changes in the protein are likely to neutralize or reduce the ability of the Ig to bind. From these tests on rats, the conclusion was arrived at that changing any of subtilisin 309 residues corresponding to 127, 128, 129, 130, 131, 151, 136, 151, 152, 153, 154, 161, 162, 163, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 186, 193, 194, 195, 196, 197, 247, 251, 261 will result in a change in the immunological potential.

PCT Publication No. WO 94/10191 discloses low allergenic proteins comprising oligomeric forms of the parent monomeric protein, wherein the oligomer has substantially retained its activity.

While some studies have provided methods of reducing the allergenicity of certain proteins and identification of epitopes which cause allergic reactions in some individuals, the assays used to identify these epitopes generally involve measurement of IgE and IgG antibody in blood sera previously exposed to the antigen. However, once an Ig reaction has been initiated, sensitization has already occurred. Accordingly, there is a need for a method of determining epitopes which cause sensitization in the first place, as neutralization of these epitopes will result in significantly less possibility for sensitization to occur, thus reducing the possibility of initial sensitization. There is also a need to produce proteins which produce an enhanced immunogenic response, and a need to identify naturally occurring proteins which produce a low immunogenic response. This invention meets these and other needs.

SUMMARY OF THE INVENTION

The present invention provides proteins which produce immunogenic responses as desired, methods of identifying and making such proteins, and methods of using such proteins. For example, as will be become apparent from the detailed description below, the methods and compositions provided herein are useful in forming hyper- and hypo-allergenic compositions. As used herein, hyper and hypo means the composition produces a greater or lesser immunogenic response, respectively, than the same composition without the proteins of the present invention. Such compositions may include cleaning compositions, textile treatments, contact lens cleaning solutions or products, peptide hydrolysis products, waste treatment products, cosmetic formulations including for skin, hair and oral care, pharmaceuticals such as blood clot removal products, research products such as enzymes and therapeutics including vaccines.

In one aspect of the invention, a polypeptide of interest is selected and provided herein. The polypeptide of interest is preferably one having a T-cell epitope and is

then varied as described below. However, polypeptides of interest may also be selected based on naturally occuring properties and not altered. Moreover, polypeptides of interest may be selected which do not have a T-cell epitope, and altered so as to have a T-cell epitope.

In one aspect of the invention provided herein is a variant of a polypeptide of interest comprising a T-cell epitope. The variant differs from the polypeptide of interest by having an altered T-cell epitope such that said variant and said polypeptide produce different immunogenic responses in an individual. The variant can be prepared and selected to produce either a greater or lesser immunogenic response than said polypeptide of interest.

The polypeptide of interest can be any polypeptide of interest. In one aspect, the polypeptide is selected from the group consisting of enzymes, hormones, factors, vaccines and cytokines. In one embodiment, the polypeptide of interest is not recognized by said individual as endogenous to said individual, or not recognized as "self". As indicated herein, the polypeptide of interest may be an enzyme. In one embodiment, the enzyme is selected from the group consisting of lipase, cellulase, endo-glucosidase H, protease, carbohydrase, reductase, oxidase, isomerase, transferase, kinase and phosphatase. In preferred embodiments, the polypeptide of interest and the variant of said polypeptide of interest each comprise at least some of the same activity. For example, if a variant of a protease is provided, said variant will produce an altered immunogenic response, but will retain detectable, and preferably comparable, protease activity.

Wherein a variant of a polypeptide of interest is provided, the T-cell epitope may be altered in a number of ways including by amino acid substitutions, deletions, additions and combinations thereof. Preferably, the T-cell epitope is altered by having amino acid substitutions. In one embodiment herein, the amino acid substitutions are made to corresponding amino acids of a homolog of the

polypeptide of interest, wherein the homolog does not comprise the same T-cell epitope in the corresponding position as the polypeptide of interest. In one aspect, the terminal portion of the polypeptide of interest comprising at least one T-cell epitope is replaced with a corresponding terminal portion of the homolog of the polypeptide of interest, wherein the replacement produces said different immunogenic response.

In another embodiment provided herein, the nucleic acids encoding the polypeptides producing the desired immunogenic response are provided herein. Moreover, the invention includes expression vectors and host cells comprising the nucleic acids provided herein. Moreover, once the polypeptides and variants thereof of the present invention are identified, substantially homologous sequences of or those sequences which hybridize to the polypeptides and variants can be identified and are provided herein. Homologous is further defined below, and can refer to similarity or identity, with identity being preferred. Preferably, the homologous sequences are amino acid sequences or nucleic acids encoding peptides having the activity of the polypeptides and variants provided herein.

In yet another aspect of the invention is a method for determining the immunogenic response produced by a protein. In one embodiment, the method comprises (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with said protein; and (d) measuring the proliferation of T-cells in said step (c).

The methods of determining immunogenic responses produced by proteins can also be used to identify comparative immunogenic responses of proteins. Therefore, in one aspect, the method of determining immunogenic responses of proteins further comprises comparing immunogenic responses of one or more proteins. The

proteins can be homologs of each other, variants of the same protein, different types of the same protein, for example, different proteases, or different peptides of the same protein.

The invention further provides a method of altering the immunogenicity of a polypeptide of interest comprising determining the immunogenicity of said polypeptide; identifying a T-cell epitope in a said polypeptide; and altering said T-cell epitope so as to alter the immunogencity of said polypeptide. As described herein, said altering can be done by altering a single amino acid or switching a portion of the polypeptide of interest with a corresponding portion of a homolog, wherein the switch produces an altered immunogenic response.

Other aspects of the invention will be understood by the skilled artisan by the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A, B1, B2 and B3 illustrate the DNA (SEQ ID:NO 1) and amino acid (SEQ ID: NO 2) sequence for *Bacillus amyloliquefaciens* subtilisin (BPN') and a partial restriction map of this gene.

Fig. 2 illustrates the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (SEQ ID:NO 3) and *Bacillus lentus* (wild-type) (SEQ ID:NO 4).

Figs. 3A and 3B illustrate an amino acid sequence alignment of subtilisin type proteases from *Bacillus amyloliquefaciens* (BPN'), *Bacillus subtilis*, *Bacillus licheniformis* (SEQ ID:NO 5) and *Bacillus lentus*. The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 illustrates the additive T-cell response of 16 peripheral mononuclear blood samples to peptides corresponding to the *Bacillus lentus* protease (GG36). Peptide E05 includes the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 5 illustrates the additive T-cell response of 10 peripheral mononuclear blood samples to peptides corresponding to the human subtilisin molecule. Peptides F10, F9, F8 and F7 all contain the amino acid sequence DQMD corresponding to the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens* in the sequence alignment of Fig. 3.

Fig. 6A and 6B/6C illustrate amino acid strings corresponding to peptides derived from the sequence of *Bacillus lentus* protease and a human subtilisin, respectively.

Fig. 7 illustrates the amino acid sequence of human subtilisin (SEQ ID:NO 6).

Fig. 8 illustrates an amino acid sequence alignment of BPN' (*Bacillus amyloliquefaciens*) protease, SAVINASE (*Bacillus lentus*) protease and human subtilisin (S2HSBT).

Fig. 9 illustrates the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 10 illustrates the T-cell response to various alanine substitutions in the E05 *Bacillus lentus* protease peptide set in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease.

Fig. 11 illustrates the T-cell response to various alanine substitutions in the E05 protease peptide (an embodiment of the T-cell epitope designated unmodified sequence) set in a sample taken from an individual known to be hypersensitive to the protease; the sequences for each peptide are also shown.

Fig. 12 illustrates the percent responders to the human subtilisin molecule.

Fig. 13A illustrates the T-cell response of peptides derived from *Humicola insolens* endogluconase (Accession number A23635). Peptides A02 and F06 represent the region corresponding to residues 70-84 and 37-51, respectively, embodiments of the T-cell epitope, of *Humicola insolens* endogluconase, wherein the full length sequence is shown in Fig.13B and A02 and F06 are shown underlined and in bold.

Fig. 14A illustrates the T-cell response to peptides derived from *Thermomyces lanuginosa* lipase (Accession number AAC08588 and PID number g2997733). Peptides B02 and C06 represent the regions corresponding to residues 83-100 and 108-121, respectively, embodiments of the T-cell epitope, of *Thermomyces lanuginosa* lipase, wherein the full length sequence is shown in Fig.14B and B02 and C06 are shown underlined and in bold.

Fig. 15A illustrates the T-cell response to peptides derived from *Streptomyces plicatus* endo-beta-N-acetylglucosaminidase. (Accession number P04067). Peptide C06 represents the region corresponding to residues 126-140, an embodiment of the T-cell epitope, of *Streptomyces plicatus* endo-beta-N-acetylglucosaminidase, wherein the full length sequence is shown in Fig.15B and C06 is shown underlined and in bold.

Fig. 16 illustrates the T-cell response to peptides derived from BPN' compiled for 22 individuals, wherein the sequences of preferred T-cell epitopes are indicated.

Fig. 17 illustrates the T-cell response to peptides derived from GG36 compiled for 22 individuals, wherein the sequences of embodiments of T-cell epitopes are indicated, GSISYPARYANAMAVGA and GAGLDIVAPGVNVQS being preferred.

Fig. 18 is an embodiment of a hybrid protein provided herein, where the N-terminus comprises N-terminal GG36 sequence and the C-terminus comprises C-terminal BPN' sequence, and wherein a comparison of the sequences with those shown in Fig. 8 indicates that the hybrid formed omits preferred T-cell epitopes of each protein.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a method for identifying T-cell epitopes is provided. Moreover, proteins including naturally occurring proteins which have relatively impotent or potent T-cell epitopes or no T-cell epitopes may be identified in accordance with the methods of the present invention. Thus, the present invention allows the identification and production of proteins which produce immunogenic responses as desired, including naturally occurring proteins as well as proteins which have been mutated to produce the appropriate response. It is understood that the terms protein, polypeptide and peptide are sometimes used herein interchangeably. Wherein a peptide is a portion of protein, the skilled artisan can understand this by the context in which the term is used.

In one embodiment, the present invention provides an assay which identifies epitopes and non-epitopes as follows: differentiated dendritic cells are combined with naïve human CD4+ and/or CD8+ T-cells and with a peptide of interest. More specifically, a method is provided wherein a T-cell epitope is recognized comprising the steps of: (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic

cells and said naïve CD4+ and/or CD8+ T-cells with a peptide of interest; (d) measuring the proliferation of T-cells in said step (c).

In one embodiment, the peptide of interest to be analyzed is derived from a polypeptide of interest. In the practice of the invention, it is possible to identify with precision the location of an epitope which can cause sensitization in an individual or sampling of individuals. In a preferred embodiment of the invention, a series of peptide oligomers which correspond to all or part of the polypeptide of interest are prepared. For example, a peptide library is produced covering the relevant portion or all of the protein. In one embodiment, the manner of producing the peptides is to introduce overlap into the peptide library, for example, producing a first peptide corresponds to amino acid sequence 1-10 of the subject protein, a second peptide corresponds to amino acid sequence 4-14 of the subject protein, a third peptide corresponds to amino acid sequence 7-17 of the subject protein, a fourth peptide corresponds to amino acid sequence 10-20 of the subject protein etc. until representative peptides corresponding to the entire molecule are created. By analyzing each of the peptides individually in the assay provided herein, it is possible to precisely identify the location of epitopes recognized by T-cells. In the example above, the greater reaction of one specific peptide than its neighbors' will facilitate identification of the epitope anchor region to within three amino acids. After determining the location of these epitopes, it is possible to alter the amino acids within each epitope until the peptide produces a different T-cell response from that of the original protein. Alternatively, the epitope may be used in its original form to stimulate an immune response against a target, e.g. infectious agent or tumor cell. Moreover, proteins may be identified herein which have desired high or low T-cell epitope potency which may be used in their naturally occurring forms.

"Antigen presenting cell" as used herein means a cell of the immune system which present antigen on their surface which is recognizable by receptors on the surface of

T-cells. Examples of antigen presenting cells are dendritic cells, interdigitating cells, activated B-cells and macrophages.

"T-cell proliferation" as used herein means the number of T-cells produced during the incubation of T-cells with the antigen presenting cells, with or without antigen.

"Baseline T-cell proliferation" as used herein means T-cell proliferation which is normally seen in an individual in response to exposure to antigen presenting cells in the absence of peptide or protein antigen. For the purposes herein, the baseline T-cell proliferation level was determined on a per sample basis for each individual as the proliferation of T-cells in response to antigen presenting cells in the absence of antigen.

"T-cell epitope" means a feature of a peptide or protein which is recognized by a T-cell receptor in the initiation of an immunologic response to the peptide comprising that antigen. Recognition of a T-cell epitope by a T-cell is generally believed to be via a mechanism wherein T-cells recognize peptide fragments of antigens which are bound to class I or class II major histocompatability (MHC) molecules expressed on antigen-presenting cells (*see e.g.*, Moeller, G. ed., "Antigenic Requirements for Activation of MHC-Restricted Responses," *Immunological Review*, Vol. 98, p. 187 (Copenhagen; Munksgaard) (1987).

"Sample" as used herein comprises mononuclear cells which are naïve, *i.e.*, not sensitized, to the antigen in question.

"Homolog" as used herein means a protein or enzyme which has similar catalytic action, structure and/or use as the protein of interest. For purposes of this invention, a homolog and a protein of interest are not necessarily related evolutionarily, *e.g.*, same functional protein from different species. It is desirable to find a homolog that has a tertiary and/or primary structure similar to the protein of interest as

replacement of the epitope in the protein of interest with an analogous segment from the homolog will reduce the disruptiveness of the change. Thus, closely homologous enzymes will provide the most desirable source of epitope substitutions. Alternatively, if possible, it is advantageous to look to human analogs for a given protein. For example, substituting a specific epitope in a bacterial subtilisin with a sequence from a human analog to subtilisin (*i.e.*, human subtilisin) should result in less allergenicity in the bacterial protein.

An "analogous" sequence may be determined by ensuring that the replacement amino acids show a similar function, the tertiary structure and/or conserved residues to the amino acids in the protein of interest at or near the epitope. Thus, where the epitope region contains, for example, an alpha-helix or a beta-sheet structure, the replacement amino acids should maintain that specific structure.

The epitopes determined according to the assay provided herein are then modified to reduce or augment the immunologic potential of the protein of interest. In a preferred embodiment, the epitope to be modified produces a level of T-cell proliferation of greater than three times the baseline T-cell proliferation in a sample. When modified, the epitope produces less than three times the baseline proliferation, preferably less than two times the baseline proliferation and most preferably less than or substantially equal to the baseline proliferation in a sample.

Preferably, the epitope is modified in one of the following ways: (a) the amino acid sequence of the epitope is substituted with an analogous sequence from a human homolog to the protein of interest; (b) the amino acid sequence of the epitope is substituted with an analogous sequence from a non-human homolog to the protein of interest, which analogous sequence produces a lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest; (c) the amino acid sequence of the epitope is substituted with a sequence which substantially mimics the major tertiary structure attributes of the epitope, but

which produces a lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest; or (d) with any sequence which produces lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest.

However, one of skill will readily recognize that epitopes can be modified in other ways depending on the desired outcome. For example, if a T-cell vaccine is desired, it is contemplated the amino acid sequence of an epitope will be substituted with amino acids which increase the immulogic response to the peptide via enhanced MHC binding and/or T-cell recognition. In another example, if altering an autoimmune response against self -antigens is desired, it is contemplated the amino acid sequence of an epitope will be substituted with amino acids that decrease or cause a shift in an inflammatory or other immune response.

The present invention extends to all proteins against which it is desired to modulate the immunogenic response, for example, peptides to be used as T-cell vaccines, or peptides or proteins to be used as therapeutic agents against, e.g., cancer, infectious diseases and autoimmune diseases. One of skill in the art will readily recognize the proteins and peptides of this invention are not necessarily native proteins and peptides. Indeed, in one embodiment of this invention, the assay described herein is used to determine the immunologic response of proteins from shuffled genes. For descriptions of gene shuffling and expression of such genes see, Stemmer, Proc. Nat'l Acad. Sci. USA 91:10747 (1994); Patten, et al., Current Opinion in Biotechnol. 8:724 (1997); Kuchner & Arnold, Trends Biotechnol. 15:523 (1997); Moore, et al., J. Mol, Biol. 272:336 (1997); Zhao, et al., Nature Biotechnol. 16:258 (1998); Giver, et al., Proc. Nat'l Acad. Sci. USA 95:12809 (1998); Harayama, Trends Biotechnol. 16:76 (1998); Lin, et al., Biotechnol., Prog. 15:467 (1999); and Sun, J. Comput. Biol. 6:77 (1999). The assay is used to predict the immunologic response of proteins encoded by shuffled genes. Once determined, the protein can be altered to modulate the immunolgic response to that protein.

In addition to the above proteins and peptides, the present invention can be used to reduce the allergenicity of proteins. These proteins include, but are not limited to, glucanases, lipases, cellulases, endo-glucosidase Hs (endo-H), proteases, carbohydrases, reductases, oxidases, isomerases, transferases, kinases, phosphatases, amylases, etc. In addition to reducing the allergenicity to an animal, such as a human, of naturally occurring amino acid sequences, this invention encompasses reducing the allergenicity of a mutated human protein, e.g., a protein that has been altered to change the functional activity of the protein. In many instances, the mutation of human proteins to e.g., increase activity, results in the incorporation of new T-cell epitope in the mutated protein. The assay of this invention can be used to determine the presence of the new T-cell epitope and determine substitute amino acids that will reduce the allergenicity of the mutated protein.

Although this invention encompasses the above proteins and many others, for the sake of simplicity, the following will describe a particularly preferred embodiment of the invention, the modification of protease. Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include a-aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

In one embodiment herein, hybrid polypeptides are provided. "Hybrid polypeptides" are proteins engineered from at least two different proteins, which are preferably homologs of one another. For example, a preferred hybrid polypeptide might have the N-terminus of a protein and the C-terminus of a homolog of the protein. In a preferred embodiment, the two terminal ends can be combined to correspond to the

full-length active protein. In a preferred embodiment, the homologs share substantial similarity but do not have identical T-cell epitopes. Therefore, in one embodiment, for example, a polypeptide of interest having one or more T-cell epitopes in the C-terminus may have the C-terminus replaced with the C-terminus of a homolog having a less potent T-cell epitope in the C-terminus, less T-cell epitopes, or no T-cell epitope in the C-terminus. Thus, the skilled artisan understands that by being able to identify T-cell epitopes among homologs, a variety of variants producing different immunogenic responses can be formed. Moreover, it is understood that internal portions, and more than one homolog can be used to produce the variants of the present invention.

More generally, the variants provided herein can be derived from the precursor amino acid sequence by the substitution, deletion, insertion, or combination thereof of one or more amino acids of the precursor amino acid sequence. Such modification is preferably of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor enzyme, but can be by the manipulation of the precursor protein. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases

both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1.

"Recombinant", "recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent 4,760,025 (RE 34,606), US Patent 5,204,015 and US Patent 5,185,258.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

"Human subtilisin" means proteins of human origin which have subtilisin type catalytic activity, e.g., the kexin family of human derived proteases. An example of such a protein is represented by the sequence in Fig. 7. Additionally, derivatives or homologs of proteins provided herein, including those from non-human sources such as mouse or rabbit, which retain the essential activity of the peptide, such as

the ability to hydrolyze peptide bonds, etc., have at least 50%, preferably at least 65% and most preferably at least 80%, more preferably at least 90%, and sometimes as much as 95 or 98% homology to the polypeptide of interest. In one embodiment, the polypeptide of interest is shown in the Figures.

The amino acid position numbers used herein refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically). "Corresponding" as used herein generally refers to an analogous position along the peptide.

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which the sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent

to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis (carlsbergensis)* and *Bacillus lentus* can be aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. The conserved residues as between BPN' and *B. lentus* are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

Thus, for example, the amino acid at position +170 is lysine (K) in both *B. amyloliquefaciens* and *B. licheniformis* subtilisins and arginine (R) in Savinase. In one embodiment of the protease variants of the invention, however, the amino acid equivalent to +170 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartic

acid (D). The abbreviations and one letter codes for all amino acids in the present invention conform to the PatentIn User Manual (GenBank, Mountain View, CA) 1990, p.101.

Homologous sequences can also be determined by using a "sequence comparison algorithm." Optimal alignment of sequences for comparison can be conducted, *e.g.,* by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection.

An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad.*

Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, an amino acid sequence is considered similar to a protein such as a protease if the smallest sum probability in a comparison of the test amino acid sequence to a protein such as a protease amino acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protein whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protein such as the protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protein such as the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protein such as a protease which may adopt a conformation such that they either

alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protein, for example, protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The variants of the present invention include the mature forms of protein variants, as well as the pro- and prepro- forms of such protein variants. The prepro- forms are the preferred construction since this facilitates the expression, secretion and maturation of the protein variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a protein which when removed results in the appearance of the "mature" form of the protein. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing

protein variants such as protease variants is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protein or to the N-terminal portion of a proprotein which may participate in the secretion of the mature or pro forms of the protein. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protein gene which participate in the effectuation of the secretion of protein under native conditions. The present invention utilizes such sequences to effect the secretion of the protein variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a protein variant consists of the mature form of the protein having a prosequence operably linked to the amino terminus of the protein and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the

invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing protein is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protein and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protein include *Bacillus subtilis* I168 (also described in US Patent 4,760,025 (RE 34,606) and US Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis, B. lentus*, etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. These techniques can be found in any molecular biology practice guide, for example, Sambrook *et al.* Molecular Cloning - A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Springs Harbor Publishing (1989) ("Sambrook"); and Current Protocols in Molecular Biology, Ausubel *et al.*(eds.), Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1997 Supplement) ("Ausubel"). Such transformed host cells are capable of either replicating vectors encoding the protein variants or expressing the desired protein variant. In the case of vectors which encode the pre- or prepro-form of the protein variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked", when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably

involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor protein may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protein of interest, preparing genomic libraries from organisms expressing the protein, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

"Hybridization" is used to analyze whether a given DNA fragment or gene corresponds to a DNA sequence described herein and thus falls within the scope of the present invention. Samples to be hybridized are electrophoresed through an agarose gel (for example, 0.8% agarose) so that separation of DNA fragments can be visualized by size. DNA fragments are typically visualized by ethidium bromide staining. The gel may be briefly rinsed in distilled H2O and subsequently depurinated in an appropriate solution (such as, for example, 0.25M HCl) with gentle shaking followed by denaturation for 30 minutes (in, for example, 0.4 M NaOH) with gentle shaking. A renaturation step may be included, in which the gel is placed in 1.5 M NaCl, 1MTris, pH 7.0 with gentle shaking for 30 minutes.

The DNA should then be transferred onto an appropriate positively charged membrane, for example, Maximum Strength Nytran Plus membrane (Schleicher & Schuell, Keene, N.H.), using a transfer solution (such as, for example, 6XSSC (900 mM NaCl, 90 mM trisodium citrate). Once the transfer is complete, generally after about 2 hours, the membrane is rinsed in e.g., 2X SSC (2X SSC = 300 mM NaCl, 30 mM trisodium citrate) and air dried at room temperature. The membrane should then be prehybridized (for approximately 2 hours or more) in a suitable

prehybridization solution (such as, for example, an aqueous solution containing per 100 mL: 20-50 mL formamide, 25 mL of 20X SSPE (1X SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaH2PO4, pH 7.7), 2.5 mL of 20% SDS, and 1 mL of 10 mg/mL sheared herring or salmon sperm DNA). As would be known to one of skill in the art, the amount of formamide in the prehybridization solution may be varied depending on the nature of the reaction obtained according to routine methods. Thus, a lower amount of formamide may result in more complete hybridization in terms of identifying hybridizing molecules than the same procedure using a larger amount of formamide. On the other hand, a strong hybridization band may be more easily visually identified by using more formamide.

A DNA probe that is complementary or is nearly complementary to the DNA sequence of interest and is generally between 100 and 1000 bases in length is labeled (using, for example, the Megaprime labeling system according to the instructions of the manufacturer) to incorporate 32P in the DNA. The labeled probe is denatured by heating to 95°C for 5 minutes and immediately added to the membrane and prehybridization solution. The hybridization reaction should proceed for an appropriate time and under appropriate conditions, for example, for 18 hours at 37°C with gentle shaking or rotating. The membrane is rinsed (for example, in 2X SSC/0.3% SDS) and then washed in an appropriate wash solution with gentle agitation. The stringency desired will be a reflection of the conditions under which the membrane (filter) is washed.

Specifically, the stringency of a given reaction (*i.e.*, the degree of homology necessary for successful hybridization) will depend on the washing conditions to which the filter is subjected after hybridization. "Low-stringency" conditions as defined herein will comprise washing a filter with a solution of 0.2X SSC/0.1% SDS at 20°C for 15 minutes. "High-stringency" conditions comprise a further washing step comprising washing the filter a second time with a solution of 0.2X SSC/0.1% SDS at 37°C for 30 minutes.

After washing, the membrane is dried and the bound probe detected. If 32P or
another radioisotope is used as the labeling agent, the bound probe can be detected
by autoradiography. Other techniques for the visualization of other probes are well-
known to those of skill. The detection of a bound probe indicates a nucleic acid
sequence has the desired homology and is encompassed within this invention.

The cloned protein is then used to transform a host cell in order to express the
protein. The protein gene is then ligated into a high copy number plasmid. This
plasmid replicates in hosts in the sense that it contains the well-known elements
necessary for plasmid replication: a promoter operably linked to the gene in
question (which may be supplied as the gene's own homologous promoter if it is
recognized, i.e., transcribed, by the host), a transcription termination and
polyadenylation region (necessary for stability of the mRNA transcribed by the host
from the protein gene in certain eucaryotic host cells) which is exogenous or is
supplied by the endogenous terminator region of the protein gene and, desirably, a
selection gene such as an antibiotic resistance gene that enables continuous
cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing
media. High copy number plasmids also contain an origin of replication for the host,
thereby enabling large numbers of plasmids to be generated in the cytoplasm
without chromosomal limitations. However, it is within the scope herein to integrate
multiple copies of the protein gene into host genome. This is facilitated by
procaryotic and eucaryotic organisms which are particularly susceptible to
homologous recombination.

In one embodiment, the gene can be a natural gene such as that from B lentus or B.
amyloliquefaciens. Alternatively, a synthetic gene encoding a naturally-occurring or
mutant precursor protein may be produced. In such an approach, the DNA and/or
amino acid sequence of the precursor protein is determined. Multiple, overlapping
synthetic single-stranded DNA fragments are thereafter synthesized, which upon
hybridization and ligation produce a synthetic DNA encoding the precursor protein.

An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protein gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protein. Such modifications include the production of recombinant proteins as disclosed in US Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of protein variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protein variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protein is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protein gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protein gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made

routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

In one aspect of the invention, the objective is to secure a variant protein having altered allergenic potential as compared to the precursor protein, since decreasing such potential enables safer use of the enzyme. While the instant invention is useful to lower allergenic potential, the mutations specified herein may be utilized in combination with mutations known in the art to result altered thermal stability and/or altered substrate specificity, modified activity or altered alkaline stability as compared to the precursor.

Accordingly, the present invention is directed to altering the capability of the T-cell epitope which includes residue positions 170-173 in *Bacillus lentus* to induce T-cell proliferation. One particularly preferred embodiment of the invention comprises making modification to either one or all of R170D, Y171Q and/or N173D. Similarly, as discussed in detail above, it is believed that the modification of the corresponding residues in any protein will result in a the neutralization of a key T-cell epitope in that protein. Thus, in combination with the presently disclosed mutations in the region corresponding to amino acid residues 170-173, substitutions at positions corresponding to N76D/S103A/V104I/G159D optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to

V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin may be used, in addition to decreasing the allergenic potential of the variant protein of the invention, to modulate overall stability and/or proteolytic activity of the enzyme. Similarly, the substitutions provided herein may be combined with mutation at the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 to Aspartate (D) in combination with the mutations S103A/V104I/G159D and optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin, to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

The most preferred embodiments of the invention include the following specific combinations of substituted residues corresponding to positions:
N76D/S103A/V104I/G159D/K170D/Y171Q/S173D;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H/Q245R;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and
V68A/N76D//S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/
Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protein.

Based on the screening results obtained with the variant proteins, the noted mutations noted above in *Bacillus amyloliquefaciens* subtilisin are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protein variants of the invention are useful in formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the protein mutants of the invention. These

include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protein variants of the present invention may be used for any purpose that native or wild-type proteins are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise, in addition to decreased allergenicity, enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteins, particularly proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% (preferably .1% to .5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteins, particularly proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described protein's denaturing temperature. In addition, proteins of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The variant proteins of the present invention can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteins of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The variants can be screened for proteolytic activity according to methods well known in the art. Preferred protease variants include multiple substitutions at positions corresponding to: N76D/S103A/V104I/G159D/K170D/Y171Q/S173D; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H/Q245R; V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin.

The proteins of this invention exhibit modified immunogenicity when compared to their precursor proteins. In preferred embodiments, the proteins exhibit reduced allergenicity. In other embodiments, the proteins exhibit increased immunogenicity. The increase in immunogenicity is manifested by an increase in B-cell or humoral immunological response, by an increase in T-cell or cellular immunological response, or by an increase in both B and T cell immunological responses. One of skill will readily recognize that the uses of the proteins of this invention will be determined, in large part, on the immunological properties of the proteins. For example, enzymes that exhibit reduced allergenicity can be used in cleaning compositions. "Cleaning compositions" are compositions that can be used to

remove undesired compounds from substrates, such as fabric, dishes, contact lenses, other solid substrates, hair (shampoos), skin (soaps and creams), etc.

Proteins, in particular, cellulases, proteases, and amylases, with reduced allergenicity can also be used in the treatment of textiles. "Textile treatment" comprises a process wherein textiles, individual yarns or fibers that can be woven, felted or knitted into textiles or garments are treated to effect a desired characteristic. Examples of such desired characteristics are "stone-washing", depilling, dehairing, desizing, softening, and other textile treatments well known to those of skill in the art.

Therapeutic proteins against which individuals mount an immune response are also included in the invention. In particular, individuals who lack endogenous production of the protein are susceptible to forming neutralizing antibodies and become refractile to treatment. Likewise, modifications of a protein may introduce new epitopes that are potentially immunogeneic. Methods of the invention can be used to identify and modify epitopes in , e.g., human Factor VIII, to prevent neutralizing responses.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents;

sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

All publications and patents referenced herein are hereby incorporated by reference in their entirety. The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

EXAMPLES

Example 1
Assay for the Identification of Peptide T-Cell Epitopes Using Naïve Human T-Cells

Fresh human peripheral blood cells were collected from "naïve" humans, *i.e.*, persons not known to be exposed to or sensitized to *Bacillus lentus* protease, for determination of antigenic epitopes in protease from *Bacillus lentus* and human subtilisin. Naïve humans is intended to mean that the individual is not known to have been exposed to or developed a reaction to protease in the past. Peripheral mononuclear blood cells (stored at room temperature, no older than 24 hours) were prepared for use as follows: Approximately 30 mls of a solution of buffy coat preparation from one unit of whole blood was brought to 50 ml with Dulbecco's phosphate buffered solution (DPBS) and split into two tubes. The samples were underlaid with 12.5 ml of room temperature lymphoprep density separation media (Nycomed density 1.077 g/ml). The tubes were centrifuged for thirty minutes at 600G. The interface of the two phases was collected, pooled and washed in DPBS. The cell density of the resultant solution was measured by hemocytometer. Viability was measured by trypan blue exclusion.

From the resulting solution, a differentiated dendritic cell culture was prepared from the peripheral blood mononuclear cell sample having a density of 108 cells per 75 ml culture flask in a solution as follows:

(1)   50 ml of serum free AIM V media (Gibco) was supplemented with a 1:100 dilution beta-mercaptoethanol (Gibco). The flasks were laid flat for two hours at 37°C in 5% CO2 to allow adherence of monocytes to the flask wall.

(2)   Differentiation of the monocyte cells to dendritic cells was as follows: nonadherent cells were removed and the resultant adherent cells (monocytes) combined with 30 ml of AIM V, 800 units/ml of GM-CSF (Endogen) and 500 units/ml of IL-4 (Endogen); the resulting mixture was cultured for 5 days under conditions at 37°C in 5% CO2. After five days, the cytokine TNFa (Endogen) was added to 0.2 units/ml, and the cytokine IL-1a (Endogen) was added to a final concentration of 50 units/ml and the mixture incubated at 37°C in 5% CO2 for two more days.

(3)   On the seventh day, Mitomycin C was added to a concentration of 50 microgram/ml was added to stop growth of the now differentiated dendritic cell culture. The solution was incubated for 60 minutes at 37°C in 5% CO2. Dendritic cells were collected by gently scraping the adherent cells off the bottom of the flask with a cell scraper. Adherent and non-adherent cells were then centrifuged at 600G for 5 minutes, washed in DPBS and counted.

(4)   The prepared dendritic cells were placed into a 96 well round bottom array at 2x104/well in 100 microliter total volume of AIM V media.

CD4+ T cells were prepared from frozen aliquots of the peripheral blood cell samples used to prepare the dendritic cells using the human CD4+ Cellect Kit (Biotex) as per the manufacturers instructions with the following modifications: the aliquots were thawed and washed such that approximately 108 cells will be applied

per Cellect column; the cells were resuspended in 4 ml DPBS and 1 ml of the Cell reagent from the Cellect Kit, the solution maintained at room temperature for 20 minutes. The resultant solution was centrifuged for five minutes at 600G at room temperature and the pellet resuspended in 2 ml of DPBS and applied to the Cellect columns. The effluent from the columns was collected in 2% human serum in DPBS. The resultant CD4+ cell solution was centrifuged, resuspended in AIMV media and the density counted.

The CD4+ T-cell suspension was resuspended to a count of 2×10^6/ml in AIM V media to facilitate efficient manipulation of the 96 well plate.

Peptide antigen is prepared from a 1M stock solution in DMSO by dilution in AIM V media at a 1:10 ratio. 10 microliters of the stock solution is placed in each well of the 96 well plate containing the differentiated dendritic cells. 100 microliter of the diluted CD4+ T-cell solution as prepared above is further added to each well. Useful controls include diluted DMSO blanks, and tetanus toxoid positive controls.

The final concentrations in each well, at 210 microliter total volume are as follows:

2×10^4 CD4+

2×10^5 dendtritic cells (R:S of 10:1)

5 mM peptide

Example 2
Identification of T-Cell Epitopes in Protease from *Bacillus lentus* and Human subtilisin

Peptides for use in the assay described in Example 1 were prepared based on the *Bacillus lentus* and human subtilisin amino acid sequence. Peptide antigens were designed as follows. From the full length amino acid sequence of either human subtilisin or *Bacillus lentus* protease provided in Figure 1, 15mers were synthetically

prepared, each 15mer overlapping with the previous and the subsequent 15mer except for three residues.

Peptides used correspond to amino acid residue strings in *Bacillus lentus* as provided in Figure 8, and peptides correspond to amino acid residues in human subtilisin as provided in Figure 7. The peptides used corresponding to the proteases is provided in Fig. 6. All tests were performed at least in duplicate. All tests reported displayed robust positive control responses to the antigen tetanus toxoid. Responses were averaged within each experiment, then normalized to the baseline response. A positive event was recorded if the response was at least 3 times the baseline response.

The immunogenic response (*i.e.,* T-cell proliferation) to the prepared peptides from human subtilisin and *Bacillus lentus* was tallied and is provided in Figures 4 and 5, respectively. T-cell proliferation was measured by the incorporated tritium method. The results shown in Figures 4 and 5 as a comparison of the immunogenic additive response in 10 individuals (Figure 4) and 16 individuals (Figure 5) to the various peptides. Response is indicated as the added response wherein 1.0 equals a baseline response for each sample. Thus, in Figure 4, a reading of 10.0 or less is the baseline response and in Figure 5 a reading of 16.0 or less the baseline response. The greater the response, the more potent the T-cell epitope is considered.

As indicated in Figures 4 and 5, the immunogenic response of the naïve blood samples from unsensitized individuals showed a marked allergenic response at the peptide fragment from *Bacillus lentus* corresponding to residues 170-173 of *Bacillus amyloliquefaciens* protease. As expected, the corresponding fragment in human subtilisin evokes merely baseline response.

Fig. 9 shows the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*. As shown in Fig. 9, the hypersensitive individual was highly responsive to the T-cell epitope represented by the peptide E05. This result confirms that, by practicing the assay according to the invention, it is possible to predict the major epitopes identified by the T-cells of a hypersensitive individual.

Fig. 10 shows the T-cell response to various alanine substitutions in the E05 peptide derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Alanine substitutions were used as substitutions for the purpose of determining the role of any specific residue within the epitope. The legend of Figure 10 refers to the position of the peptide in which an alanine was substituted, i.e., in peptide E06 (sequence GSISYPARYANAMAV), G to A = 2, S to A = 3, I to A = 4, S to A = 5, Y to A = 6, P to A = 7, R to A = 8, Y to A = 9, N to A = 10, M to A = 11 and V to A = 12. As indicated in Figure 10, substitution of either of the residues R170A, Y171A and/or N173A in protease from *Bacillus lentus* results in dramatically reduced response in the hypersensitive individual's blood sample.

From these results, it is apparent that the residues 170, 171 and 173 are largely responsible for the initiation of allergic reaction within the protease from *Bacillus lentus*.

Example 3

Identification of T-Cell Epitopes in Cellulase
from *Humicola insolens* (Carezyme®)

The procedure described above was performed on peptides derived from a cellulase from *Humicola insolens* (Carezyme ® from Novo Nordisk). As can be seen from Figure 13, 2 T-cell epitopes were discovered, A01 and F06.

Example 4

Identification of T-Cell Epitopes in Lipase from *Thermomyces Lanuginosa*
(Lipolase®)

The procedure described in Example 2 was performed on peptides derived from a lipase from *Thermomyces lanuginosa* (Lipolase ® from Novo Nordisk). As can be seen from Figure 14, two T-cell epitopes were discovered, A12 and C06. Peptide E03 effected slightly increased T-cell proliferation in the naïve donors, however, this peptide is consecutive to A12 and they represent one epitope. In this regard, the skilled artisan understands that the length of the epitopes can be varied, and the precise potency of the epitope, naturally occuring or mutated can be determined by the methods herein.

Example 5

Identification of T-Cell Epitopes in Endoglucanase H from Streptomyces plicatus

The procedure described in Example 2 was performed on peptides derived from endoglucanase H from *Streptomyces plicatus*. As can be seen from Figure 15, a single T-cell epitope was discovered, C06.

Example 6

Identification of T-Cell Epitopes in a Protease Hybrid (GG36-BPN')

After determining the location of a T-cell epitope, a protease hybrid was constructed using established protein engineering techniques. The hybrid was constructed so that a highly allergenic amino acid sequence of the protein was replaced with a corresponding sequence from a less allergenic homolog. In this instance, the first 122 amino acids of the protease were derived from GG36, and the remaining amino acid sequence was derived from BPN'.

The hybrid was first tested from a 100 ppm sample in North American condition in 24 well assay at .5 ppm, superfixed swatches, liquid (Tide KT) at .5 in 24 well assay with 3K swatches, and in the N'N'-dimethyl Casein Assay, 5 g/l DMC in NA detergent, TNBS dectection method.

The results are shown in Figures 16, 17 and 18.

Example 7

Identification of a Naturally Occuring Low Immunogenic Protein

Using the methods herein, proteinase K was identified as producing a lower immunogenic response than other commercially available proteases. Proteinase K as identified herein is from Tritirachium Album limber. For a general description of proteases and methodologies, see, Mathew, C.G.P. Isolation of high molecular weight eukaryotic DNA, in Methods in Molecular Biology, vol. 2: Nucleic Acids (Walker, J.M.,ed.), Humana, Clifton, NJ, (1984) pp. 31-34.

WE CLAIM:

1. A variant of a polypeptide of interest comprising a T-cell epitope, wherein said variant differs from said polypeptide of interest by having an altered T-cell epitope such that said variant and said polypeptide produce different immunogenic responses in an individual.

2. The variant of claim 1 wherein said immunogenic response produced by said variant is less than said immunogenic response produced by said polypeptide of interest.

3. The variant of claim 1 wherein said immunogenic response produced by said variant is greater than said immunogenic response produced by said polypeptide of interest.

4. The variant of claim 1 wherein said polypeptide of interest is selected from the group consisting of enzymes, hormones, factors, vaccines and cytokines.

5. The variant of claim 1 wherein said polypeptide of interest is not recognized by said individual as endogenous to said individual.

6. The variant of claim 1 wherein said polypeptide of interest is an enzyme selected from the group consisting of lipase, cellulase, endo-glucosidase H, protease, carbohydrases, reductase, oxidase, isomerase, transferase, kinase and phosphatase.

7. The variant of claim 1 wherein said T-cell epitope is altered with amino acid substitutions.

8. The variant of claim 1 wherein said T-cell epitope is altered by having a terminal portion of said polypeptide of interest comprising said T-cell epitope replaced with a corresponding terminal portion of a homolog of said polypeptide of interest wherein said homolog does not comprise a T-cell cell epitope identical to said replaced T-cell epitope.

9. The variant of claim 8 wherein said variant comprises at least one less T-cell epitope than said polypeptide of interest and said homolog combined.

10. The variant of claim 8 wherein said variant comprises at least two less T-cell epitopes than said polypeptide of interest and said homolog combined.

11. A nucleic acid encoding the variant of claim 1.

12. An expression vector comprising the nucleic acid of claim 11.

13. A host cell transformed with the expression vector of claim 12.

14. A cleaning composition, an animal feed composition, or a composition for treating a textile comprising the variant of claim 6.

15. The variant of claim 1 further comprising a pharmaceutically acceptable carrier.

16. A cleaning composition, an animal feed composition, or a composition for treating a textile comprising a naturally occuring enzyme producing a reduced immunogenic response in comparison to another enzyme of the same type.

17. The composition of claim 16 wherein said type is a protease.

18. The composition of claim 16 wherein said enzyme is proteinase K.

19. A method for determining the immunogenic response produced by a protein, comprising:

(a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells;

(b) promoting differentiation in said solution of dendritic cells;

(c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with said protein; and

(d) measuring the proliferation of T-cells in said step (c).

20. The method of claim 19 further comprising comparing said immunogenic response to another protein.

21. The method of claim 20 wherein said protein and said another protein are homologs of one another.

22. The method of claim 20 wherein said protein and said another protein are each proteases.

23. The method of claim 20 wherein said protein and said another protein are each different peptides of the same protein.

24. A method of altering the immunogenicity of a polypeptide of interest comprising:

a) determining the immunogenicity of said polypeptide;

b) identifying a T-cell epitope in a said polypeptide; and

c) altering said T-cell epitope so as to alter the immunogencity of said polypeptide.

25. The method of claim 24 wherein said T-cell epitope is altered by having at least one amino acid substitution.

26. The method of claim 25 wherein said amino acid substitution is made by altering a nucleic acid encoding for said T-cell epitope.

27. The method of claim 24 wherein said T-cell epitope is altered by replacing a portion of said polypeptide of interest comprising said T-cell epitope with a corresponding portion of a homolog of said polypeptide of interest, where said corresponding portion does not contain said T-cell epitope.

28. The method of claim 27 wherein said portion is a terminal portion of said polypeptide of interest.

Abstract

The present invention relates to a novel methods and compositions for producing hyper and hypo allergenic compositions. Specifically, the present invention comprises neutralizing or reducing the ability of T-cells to recognize epitopes and thus prevent sensitization of an individual to the protein. Alternatively, T-cell epitopes are mutated to produce increased immunogenic reactions. Moreover, naturally occuring proteins are provided.

Eco RI ... *Cla* I *Pvu* II ... *Bam* HI

P RBS —PRE —PRO MAT TERM

◄————————————————— 1.5kb —————————————————►

FIG._1A

⑤ ③ ④

P   RBS   -107
                Met

1 GGTCTACTAAAATATTATTCCATACTATACAATTAATACACAGAATAATCTGTCTATTGGTTATTCTGCAATGAAAAAAGGAGGATTAAAGA GTG

               -100   PRE   -90

	-100	PRE		-90			-80			-70	PRO		-60		Met

99   Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
     AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC

174   Ser Ala Gln Ala Ala Ala Lys Asn Gly Glu Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
     TCT GCC CAG GCG GCA GCA AAG AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG CAA TTC AGC ACG ATG

249   Ser Ala Ala Ala Lys Glu Ile Ile Ser Glu Glu Lys Lys Lys Val Lys Lys Asp Pro Ser Val Ala Tyr Val Glu
     AGC GCC GCT GCG GCG AAG GAA ATC ATT TCT GAA AAA GGG GGG AAA GTG AAA GAC CCG AGC GTC GCT TAT GTT GAA

324   Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp
     GCT TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC GAC AGC GTC GCT TAC GTT GAA GAA GAT

399   His Val Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln
     CAC GTA GCA CAT GCT TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA

474   Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser His Pro Asp Leu Lys Val Val
     GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT GAC AGC GGT ATC GAT TCT CAT CCT GAT CTT AAG TTA GTA

MAT
→
-1   1

     -50   -40   -30   -20   -10   10   20   30   40

FIG._1B - 1

Ala Gly Gly Ala Gly Ser Pro Val   50 Met Ser His Ala Asn Ser Asp Asn Gln   60 Gln Asp Asn Asn Ser His Thr Gly His Val Ala
549 GCA GGC GGA GCC GGT TCT CCT GTT ATG AGC CAC GCA AAC TCT GAC AAC CAA GAC AAC AAC TCT CAC ACT GGA CAC GTT GCC

Gly Thr Val Leu Gly Ala Ala Ala Asp Ala Asp   70 Asn Met Asn Ser   80 Gly Ile Ser Tyr Gln   90 Leu Ala Ser Tyr Ala Asn Val Lys
624 GGC ACA GTT CTC GGT GCC GCT GCA GAC GCT GAT AAC ATG AAC AGC GGT ATC TCA TAC CAA CTT GCA TCA TAC GCA AAT GTA AAA

Val Leu Gly Ile Ile Asn Gly Val Ile Trp Ser Gln Gly Gly Ala Ala Ala   100 Glu Ile Trp Ala Ala Ala Ala   110 Asn Ala Ala Asn Met Met
699 GTT CTC GGT ATT ATC AAC GGT GTA ATC TGG AGC CAA GGT GGG GCA GCC GCG GAG ATC TGG GCG GCA GCA GCA AAC GCA GCG AAC AAT ATG

Asp Val Ile Asn Ser Val Met Val Leu Ser   120 Gly Val Pro Gly Ala Ala   130 Gly Ser Gln Ile Ser   140 Lys Asp Lys Ala Val Ala
774 GAC GTT ATT AAC AGC GTT ATG GTT CTC AGC GGT GTA CCT GGC GCA GCA GGT TCT CAA ATC TCT AAA GAT AAA GCC GTT GCA

Ser Gly Val Val Val Ala Met Ala Val Ser Glu Asn Gly Asn Asp Ser Ser Ser Asn Ser Ser   150 Thr Thr Ser Ser   160 Ser Thr Val Thr Ser Val Gly
849 TCC GGC GTC GTA GTC GTT ATG GCG GTT AGC GAA AAC GGT AAC GAC TCC AGC TCA AGC AGC TCC ACA AGC AGC AGC ACA GTG ACA GGG GGT

Lys Tyr Pro Ser Ile Val Gly Pro Gly Val Asp Ser Asn Gln Arg Ala Arg   170 Ser Asn Gln   180 Ala Ser Phe Lys Tyr   190 Ser Val Gly Pro
924 AAA TAC CCT TCT ATT GTT GGC CCT GGC GTA GAT AGC AAC CAA AGA GCA AGA TCC AGC CAA GCA GCA TCT TTC AAA TAC TCA GTA GGA CCT

Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Ser Thr Leu Ala Gln Ser Thr Leu Gly Asn Gly Tyr Ala   210 Tyr Tyr Asn Gly
999 GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC AGC ACG CTT GCA CAA AGC ACG CTT GGA AAC GGG TAC GCG CCG TAC AAC GGT

Thr Ser Met Ala Ser Pro His Val   220 Ala Ala Ala Ala Leu Ile Leu   230 Ser Lys His Pro Lys Ser Leu Trp Asn   240 Thr Asn Thr
1074 ACG TCA ATG GCA TCT CCG CAC GTT GCC GCG GCG GCT TTG ATT CTT TCT AAG CAC CCG AAG AGT CTT TGG AAC ACA AAC ACT

FIG._1B-2

                              250   260
Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
1149 CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA CTT GGT GAT TCT TTC TAC TAC GGA AAA GGG CTG ATC AAC

                                                    TERM
270   275   OC
Val Gln Ala Ala Ala Gln Ala   Gln OC
1224 GTA CAG GCG GCA GCT CAG GCT CAG TAA AACATAAAAACCGGCCTTGCCCCGCCCGGGTTTTTATTTTTCTTCCTCCGGCATGTTCAATCGCGTCC

1316 ATAATCGACCGATGGCTCCCTCTGAAAATTTAACGACGAACGCGCGGGTTGACCCGGCTCAGTCCGTAGCGGCCAAGTCCTGAACGTCTCAATCGCGC

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCGGTAACGGTCGGCGGCGGTTTTCTGTATACCGGGAGACGGCGCATTCGGTAATCGGATC

FIG._1B - 3

FIG._1B

FIG._1B - 1
FIG._1B - 2
FIG._1B - 3

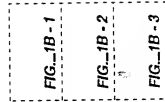CONSERVED RESIDUES IN SUBTILISINS FROM
BACILLUS AMYLOLIQUEFACIENS

```
1               10                    20
A Q S V P . G . . . . . A P A . H . . G

21              30                    40
. T G S . V K V A V . D . G . . . . H P

41              50                    60
D L . . . G G A S . V P . . . . . . Q D

61              70                   . 80
. N . H G T H V A G T . A A L N N S I G

81              90                   100
V L G V A P S A . L Y A V K V L G A . G

101             110                  120
S G . . S . L . . G . E W A . N . . . .

121             130                  140
V . N . S L G . P S . S . . . . . A . .

141             150                  160
. . . . . G V . V V A A . G N . G . . .

161             170                  180
. . . . . . Y P . . Y . . . . A V G A .

181             190                  200
D . . N . . A S F S . . G . . L D . . A

201             210                  220
P G V . . Q S T . P G . . Y . . . N G T

221             230                  240
S M A . P H V A G A A A L . . . K . . .

241             250                  260
W . . . Q . R . . L . N T . . . L G . .

261             270
. . Y G . G L . N . . A A . .
```

FIG._2

COMPARISON OF SUBTILISIN SEQUENCES FROM:

B.amyloliquefaciens
B.subtilis
B.licheniformis
B.lentus

```
                10         20         30
01
A Q S V P Y G V S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q T V P Y G I P L I K A D K V Q A Q G F K G A N V K V A V L D T G I Q A S H P
A Q S V P W G I S R V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P

                50         60         70
41
D L K V A G G A S M V P S E T N P F Q D N N S H G T H V A G T V A A L N N S I G
D L N V R G G A S F V P S E T N P Y Q D G S S H G T H V A G T I A A L N N S I G
D L N V V G G A S F V A G E A Y N * T D G N G H G T H V A G T V A A L D N T T G
D L N I R G G A S F V P G E * P S T Q D G N G H G T H V A G T I A A L N N S I G

                90        100        110
81
V L G V A P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I A N N M D
V L G V S P S A S L Y A V K V L D S T G S G Q Y S W I I N G I E W A I S N N M D
V L G V A P S V S L Y A V K V L N S S G S G S Y S G I V S G I E W A T T N G M H
V L G V A P S A E L Y A V K V L G A S G S G S V S S I A Q G L E W A G N N G M H

               130        140        150
121
V I N M S L G G P S G S A A L K A A V D K A V A S G V V V V A A A G N E G T S G
V I N M S L G G P T G S T A L K T V V D K A V S S G I V V A A A A G N E G S S G
V I N M S L G G P T G S T A M K Q A V D N A Y A R G V V V V A A A G N S G N S G
V A N L S L G S P S P S A T L E Q A V N S A T S R G V L V V A A S G N S G A G S
```

FIG._3A

```
161  SSTVGYPGKKYPSVIAVGAVDSSNQRASPSSVGPELDVMA
     SSTVGYPAKKYPSTIAVGAVNSNQRASFSSAGSELDVMA
     STNT*ISYPARYANAMAVGATDQNNRASFSQYGAGLDIVA
     **
            170        180        190

201  PGVSHIQSTLPGGNKIYGAYNGTSMASPHVAGAAALILSKHPN
     PGVSHIQSTLPGGTYIYCAYNGTSMATPHVAGAAALILSKHPT
     PGAGVYSSTYPTNTYYATLNGTSMATPHVAGAAALILSKHPN
     PGVNVQSTYPGSTYYASLNGTSMATPHVAGAAALVKQKNPS
            210        220        230

241  WTNTQVRSLENTTTKLGDSFYYGKGLIINVQAAAQ
     WTNAQVRDRLESTATYLGSSFYYGKGLIHNVQAAAQ
     LSASQVRRLSSSTATYLGSSFYIYGSGLVNVAEAATR
     WSNVQIRNHLKNTATSLGSTNLIYGSGLV NVAAATR
            250        260        270
```

FIG._3B

FIG._3A

FIG._3B

FIG._3

FIG. 4

FIG. 5

1	A12	IKDFHVYFRESRDAG
2	A11	LEQAVNSATSRGVLV
3	A10	AQSVPWGISRVQAPA
4	A9	VPWGISRVQAPAAHN
5	A8	GISRVQAPAAHNRGL
6	A7	RVQAPAAHNRGLTGS
7	A6	APAAHNRGLTGSGVK
8	A5	AHNRGLTGSGVKVAV
9	A4	RGLTGSGVKVAVLDT
10	A3	TGSGVKVAVLDTGIS
11	A2	GVKVAVLDTGISTHP
12	A1	VAVLDTGISTHPDLN
13	B12	LDTGISTHPDLNIRG
14	B11	GISTHPDLNIRGGAS
15	B10	THPDLNIRGGASFVP
16	B9	DLNIRGGASFVPGEP
17	B8	IRGGASFVPGEPSTQ
18	B7	GASFVPGEPSTQDGN
19	B6	FVPGEPSTQDGNGHG
20	B5	GEPSTQDGNGHGTHV
21	B4	STQDGNGHGTHVAGT
22	B3	DGNGHGTHVAGTIAA
23	B2	GHGTHVAGTIAALNN
24	B1	THVAGTIAALNNSIG
25	C12	AGTIAALNNSIGVLG
26	C11	IAALNNSIGVLGVAP
27	C10	LNNSIGVLGVAPSAE
28	C9	SIGVLGVAPSAELYA
29	C8	VLGVAPSAELYAVKV
30	C7	VAPSAELYAVKVLGA
31	C6	SAELYAVKVLGASGS
32	C5	LYAVKVLGASGSGSV
33	C4	VKVLGASGSGSVSSI
34	C3	LGASGSGSVSSIAQG
35	C2	SGSGSVSSIAQGLEW
36	C1	GSVSSIAQGLEWAGN
37	D12	SSIAQGLEWAGNNGM
38	D11	AQGLEWAGNNGMHVA
39	D10	LEWAGNNGMHVANLS
40	D9	AGNNGMHVANLSLGS
41	D8	NGMHVANLSLGSPSP
42	D7	HVANLSLGSPSPSAT
43	D6	NLSLGSPSPSATLEQ
44	D5	LGSPSPSATLEQAVN
45	D4	PSPSATLEQAVNSAT
46	D3	SATLEQAVNSATSRG
47	D2	LEQAVNSATSRGVLV
48	D1	AVNSATSRGVLVVAA
49	E12	SATSRGVLVVAASGN
50	E11	SRGVLVVAASGNSGA
51	E10	VLVVAASGNSGAGSI
52	E9	VAASGNSGAGSISYP
53	E8	SGNSGAGSISYPARY
54	E7	SGAGSISYPARYANA
55	E6	GSISYPARYANAMAV
56	E5	SYPARYANAMAVGAT
57	E4	ARYANAMAVGATDQN
58	E3	ANAMAVGATDQNNNR
59	E2	MAVGATDQNNNRASF
60	E1	GATDQNNNRASFSQY
61	F12	DQNNNRASFSQYGAG
62	F11	NNRASFSQYGAGLDI
63	F10	ASFSQYGAGLDIVAP
64	F9	SQYGAGLDIVAPGVN
65	F8	GAGLDIVAPGVNVQS
66	F7	LDIVAPGVNVQSTYP
67	F6	VAPGVNVQSTYPGST
68	F5	GVNVQSTYPGSTYAS
69	F4	VQSTYPGSTYASLNG
70	F3	TYPGSTYASLNGTSM
71	F2	GSTYASLNGTSMATP
72	F1	YASLNGTSMATPHVA
73	G12	LNGTSMATPHVAGAA
74	G11	TSMATPHVAGAAALV
75	G10	ATPHVAGAAALVKQK
76	G9	HVAGAAALVKQKNPS
77	G8	GAAALVKQKNPSWSN
78	G7	ALVKQKNPSWSNVQI
79	G6	KQKNPSWSNVQIRNH
80	G5	NPSWSNVQIRNHLKN
81	G4	WSNVQIRNHLKNTAT
82	G3	VQIRNHLKNTATSLG
83	G2	RNHLKNTATSLGSTN
84	G1	LKNTATSLGSTNLYG
85	H12	TATSLGSTNLYGSGL
86	H11	SLGSTNLYGSGLVNA
87	H10	STNLYGSGLVNAEAA
88	H9	NLYGSGLVNAEAATR

FIG. 6A

1	A12	IKDFHVYFRESRDAG
2	A11	DAELHIFRVFTNNQV
3	A10	PLRRASLSLGSGFWH
4	A9	RASLSLGSGFWHATG
5	A8	LSLGSGFWHATGRHS
6	A7	GSGFWHATGRHSSRR
7	A6	FWHATGRHSSRRLLR
8	A5	ATGRHSSRRLLRAIP
9	A4	RHSSRRLLRAIPRQV
10	A3	SRRLLRAIPRQVAQT
11	A2	LLRAIPRQVAQTLQA
12	A1	AIPRQVAQTLQADVL
13	B12	RQVAQTLQADVLWQM
14	B11	AQTLQADVLWQMGYT
15	B10	LQADVLWQMGYTGAN
16	B9	DVLWQMGYTGANVRV
17	B8	WQMGYTGANVRVAVF
18	B7	GYTGANVRVAVFDTG
19	B6	GANVRVAVFDTGLSE
20	B5	VRVAVFDTGLSEKHP
21	B4	AVFDTGLSEKHPHFK
22	B3	DTGLSEKHPHFKNVK
23	B2	LSEKHPHFKNVKERT
24	C12	KHPHFKNVKERTNWT
25	C12	HFKNVKERTNWTNER
26	C11	NVKERTNWTNERTLD
27	C10	ERTNWTNERTLDDGL
28	C9	NWTNERTLDDGLGHG
29	C8	NERTLDDGLGHGTFV
30	C7	TLDDGLGHGTFVAGV
31	C6	DGLGHGTFVAGVIAS
32	C5	GHGTFVAGVIASMRE
33	C4	TFVAGVIASMRECQG
34	C3	AGVIASMRECQGFAP
35	C2	IASMRECQGFAPDAE
36	C1	MRECQGFAPDAELHI
37	D12	CQGFAPDAELHIFRV
38	D11	FAPDAELHIFRVFTN
39	D10	DAELHIFRVFTNNQV
40	D9	LHIFRVFTNNQVSYT
41	D8	FRVFTNNQVSYTSWF
42	D7	FTNNQVSYTSWFLDA
43	D6	NQVSYTSWFLDAFNY
44	D5	SYTSWFLDAFNYAIL
45	D4	SWFLDAFNYAILKKI
46	D3	LDAFNYAILKKIDVL
47	D2	FNYAILKKIDVLNLS
48	D1	AILKKIDVLNLSIGG

49	E12	KKIDVLNLSIGGPDF
50	E11	DVLNLSIGGPDFMDH
51	E10	NLSIGGPDFMDHPFV
52	E9	IGGPDFMDHPFVDKV
53	E8	PDFMDHPFVDKVWEL
54	E7	MDHPFVDKVWELTAN
55	E6	PFVDKVWELTANNVI
56	E5	DKVWELTANNVIMVS
57	E4	WELTANNVIMVSAIG
58	E3	TANNVIMVSAIGNDG
59	E2	NVIMVSAIGNDGPLY
60	E1	MVSAIGNDGPLYGTJ
61	F12	AIGNDGPLYGTLNNP
62	F11	NDGPLYGTLNNPADQ
63	F10	PLYGTLNNPADQMDV
64	F9	GTLNNPADQMDVIGV
65	F8	NNPADQMDVIGVGGI
66	F7	ADQMDVIGVGGIDFE
67	F6	MDVIGVGGIDFEDNI
68	F5	IGVGGIDFEDNIARF
69	F4	GGIDFEDNIARFSSR
70	F3	DFEDNIARFSSRGMT
71	F2	DNIARFSSRGMTTWE
72	F1	ARFSSRGMTTWELPG
73	G12	SSRGMTTWELPGGYG
74	G11	GMTTWELPGGYGRMK
75	G10	TWELPGGYGRMKPDI
76	G9	LPGGYGRMKPDIVTY
77	G8	GYGRMKPDIVTYGAG
78	G7	RMKPDIVTYGAGVRG
79	G6	PDIVTYGAGVRGSGV
80	G5	VTYGAGVRGSGVKGG
81	G4	GAGVRGSGVKGGCRA
82	G3	VRGSGVKGGCRALSG
83	G2	SGVKGGCRALSGTSV
84	G1	KGGCRALSGTSVASP
85	H12	CRALSGTSVASPVVA
86	H11	LSGTSVASPVVAGAV
87	H10	TSVASPVVAGAVTLL
88	H9	ASPVVAGAVTLLVST
89	H8	VVAGAVTLLVSTVQK
90	H7	GAVTLLVSTVQKREL
91	H6	TLLVSTVQKRELVNP
92	H5	VSTVQKRELVNPASM
93	H4	VQKRELVNPASMKQA
94	H3	RELVNPASMKQALIA
95	H2	VNPASMKQALIASAR
96	H1	ASMKQALIASARRLP

FIG. 6B

97	I12	IKDFHVYFRESRDAG
98	I11	DAELHIFRVFTNNQV
99	I10	KQALIASARRLPGVN
100	I9	LIASARRLPGVNMFE
101	I8	SARRLPGVNMFEQGH
102	I7	RLPGVNMFEQGHGKL
103	I6	GVNMFEQGHGKLDLL
104	I5	MFEQGHGKLDLLRAY
105	I4	QGHGKLDLLRAYQIL
106	I3	GKLDLLRAYQILNSY
107	I2	DLLRAYQILNSYKPQ
108	I1	RAYQILNSYKPQASL
109	J12	QILNSYKPQASLSPS
110	J11	NSYKPQASLSPSYID
111	J10	KPQASLSPSYIDLTE
112	J9	ASLSPSYIDLTECPY
113	J8	SPSYIDLTECPYMWP
114	J7	YIDLTECPYMWPYCS
115	J6	LTECPYMWPYCSQPI
116	J5	CPYMWPYCSQPIYYG

FIG. 6C

```
MKLVNIWLLLLVVLLCGKKHLGDRLEKKSFEKAPCPGCSHLTLKVEFSSTVVEYEYIVAFNGYFT
AKARNSFISSALKSSEVDNWRIIPRNNPSSDYPSDFEVIQIKEKQKAGLLTLEDHPNIKRVTPQR
KVFRSLKYAESDPTVPCNETRWSQKWQSSRPLRRASLSLGSGFWHATGRHSSRRLLRAIPRQVAQ
TLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERTNWTNERTLDDGLGHGTFVAGVIASM
RECQGFAPDAELHIFRVFTNNQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWEL
TANNVIMVSAIGNDGPLYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTWELPGGYGRMKPD
IVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLVSTVQKRELVNPASMKQALIASARRLPG
VNMFEQGHGKLDLLRAYQILNSYKPQASLSPSYIDLTECPYMWPYCSQPIYYGGMPTVVNVTILN
GMGVTGRIVDKPDWQPYLPQNGDNIEVAFSYSSVLWPWSGYLAISISVTKKAASWEGIAQGHVMI
TVASPAETESKNGAEQTSTVKLPIKVKIIPTPPRSKRVLWDQYHNLRYPPGYFPRDNLRMKNDPL
DWNGDHIHTNFRDMYQHLRSMGYFVEVLGAPFTCFDASQYGTLLMVDSEEEYFPEEIAKLRRDVD
NGLSLVIFSDWYNTSVMRKVKFYDENTRQWWMPDTGGANIPALNELLSVWNMGFSDGLYEGEFTL
ANHDMYYASGCSIAKFPEDGVVITQTFKDQGLEVLKQETAVVENVPILGLYQIPAEGGGRIVLYG
DSNCLDDSHRQKDCFWLLDALLQYTSYGVTPPSLSHSGNRQRPPSGAGSVTPERMEGNHLHRYSK
VLEAHLGDPKPRPLPACPRLSWAKPQPLNETAPSNLWKHQKLLSIDLDKVVLPNFRSNRPQVRPL
SPGESGAWDIPGGIMPGRYNQEVGQTIPVFAFLGAMVVLAFFVVQINKAKSRPKRRKPRVKRPQL
MQQVHPPKTPSV
```

FIG. 7

```
              10         20         30         40         50
BPN'     AQSVPYGVSQ-IKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLK-VAGGA  48
SAVINASE AQSVPWGISR-VQAPAAHNRGLTGSGVKVAVLDTGI-STHPDLN-IRGGA  47
S2HSBT   -RAIPRQVAQTLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERT  49


              60         70         80         90        100
BPN'     SMVPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGA  98
SAVINASE SFVPGEPST-QDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGA  96
S2HSBT   NW--TNERTLDDGLGHGTFVAGVIASMRECQGF---APDAELHIFRVFTN  94


             110        120        130        140        150
BPN'     DGSGQYSWIINGIEWAIANNMDVINMSLGGPS-GSAALKAAVDKAVASGV 147
SAVINASE SGSGSVSSIAQGLEWAGNNGMHVANLSLGSPS-PSATLEQAVNSATSRGV 145
S2HSBT   NQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWELTANNV 144


             160        170        180        190        200
BPN'     VVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPEL- 197
SAVINASE LVVAASGNSGA----GSISYPARYANAMAVGATDQNNNRASFSQYGAGL- 191
S2HSBT   IMVSAIGNDGP--LYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTW 192


             210        220        230        240        250
BPN'     ----------DVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALIL 235
SAVINASE ----------DIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVK 229
S2HSBT   ELPGGYGRMKPDIVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLV 242


             260        270        280        290
BPN'     SKHPNWTNTQ---VRSSLENTTTKLGDSFYYGKGLINVQAAAQ      275
SAVINASE QKNPSWSNVQ---IRNHLKNTATSLGSTNLYGSGLVNAEAATR      269
S2HSBT   STVQKRELVNPASMKQALIASARRLPGVNMFEQG-----HGKL      280
```
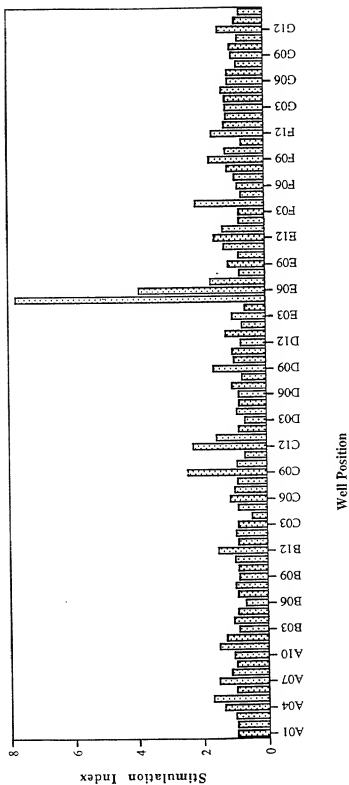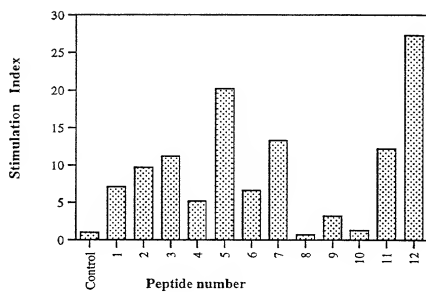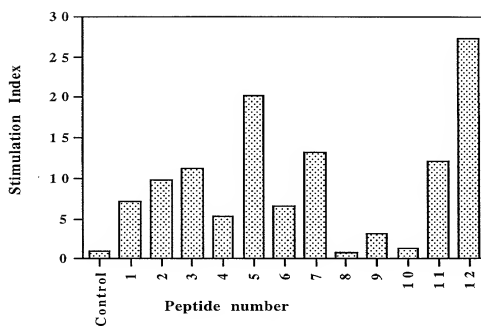
FIG. 8

FIG. 9

Well Position

Stimulation Index

FIG. 10

Peptide number	Sequence
1 (unmodified sequence)	GSISYPARYANAMAV
2	ASISYPARYANAMAV
3	GAISYPARYANAMAV
4	GSASYPARYANAMAV
5	GSIAYPARYANAMAV
6	GSISAPARYANAMAV
7	GSISYAARYANAMAV
8	GSISYPAAYANAMAV
9	GSISYPARAANAMAV
10	GSISYPARYAAAMAV
11	GSISYPARYANAAAV
12	GSISYPARYANAMAA

FIG. 11

Human subtilisin percent responders
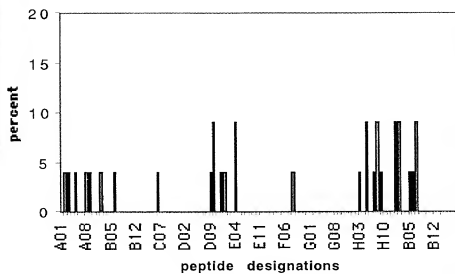
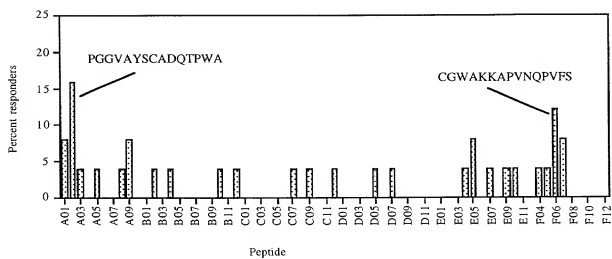FIG. 12

FIG. 13A

```
            1          2          3          4          5
   1234567890 1234567890 1234567890 1234567890 1234567890
   MRSSPLLPSA VVAALPVLAL AADGRSTRYW DCCKPSCGWA KKAPVNQPVF
   SCNANFQRIT DFDAKSGCEP GGVAYSCADQ TPWAVNDDFA LGFAATSIAG
   SNEAGWCCAC YELTFTSGPV AGKKMVVQST STGGDLGSNH FDLNIPGGGV
   GIFDGCTPQF GGLPGQRYGG ISSRNECDRF PDALKPGCYW RFDWFKNADN
   PSFSFRQVQC PAELVARTGC RRNDDGNFPA VQIPSSSTSS PVNQPTSTST
   TSTSTTSSPP VQPTTPSGCT AERWAQ
```
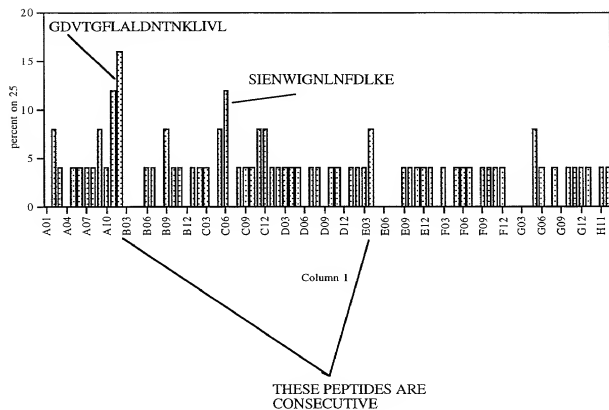
FIG. 13B

FIG. 14A

```
  1 mrsslvlffv sawtalaspi rrevsqdlfn qfnlfaqysa aaycgknnda
 51 pagtnitctg nacpevekad atflysfeds gvgdvtqfla ldntnklivl
101 sfrgsrsien wiqnlnfdlk eindicsgcr ghdgftsswr svadtlrqkv
151 edavrehpdy rvvftghslg galatvagad lrgngydidv fsygaprvgn
201 rafaefltvq tggtlyrith tndivprlpp refgyshssp eywiksgtlv
251 pvtrndivki egidatggnn qpnipdipah lwyfgligtc l
```
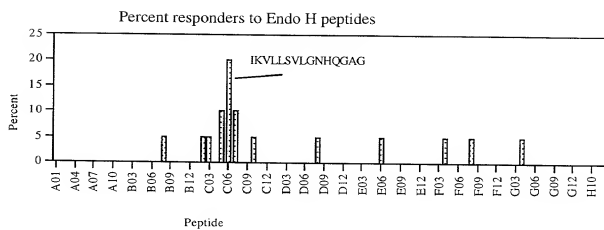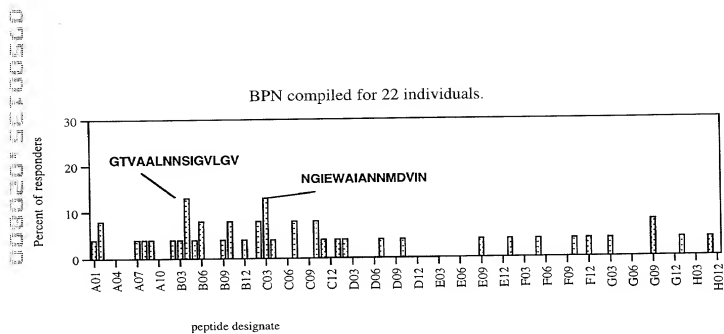
FIG. 14B

FIG. 15A

```
  1 mftpvrrrvr taalalsaaa alvlgstaas gasatpspap apapapvkqg
 51 ptsvayvevn nnsmlnvgky tladgggnaf dvavifaani nydtgtktay
101 lhfnenvqrv ldnavtqirp lqqqgikvll svlgnhqgaq fanfpsqqaa
151 safakqlsda vakygldgvd fddeyaeygn ngtaqpndss fvhlvtalra
201 nmpdkiisly nigpaasrls yggvdvsdkf dyawnpyygt wqvpgialpk
251 aqlspaavei grtsrstvad larrtvdegy gvyltynldg gdrtadvsaf
301 trelygseav rtp
```

FIG. 15B

FIG. 16

FIG. 17

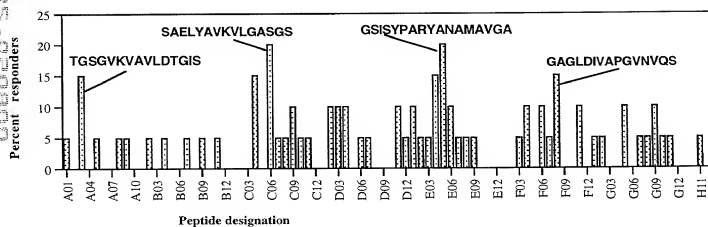Hybrid enzyme sequence (GG36-BPN)

GG36
AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGH

                                                                    BPN

GTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVINMSLGGS
                                                              △

GSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGP

ELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTTTKLGD

SFYY GKGLINVQAAAQ

FIG. 18